Engineering a Therapeutic Lectin: Uncoupling Mitogenicity from Antiviral Activity

Supplemental Experimental Procedures and Supplemental References

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Supplemental Experimental Procedures

Lectins and carbohydrates

Methyl α-D-mannopyranoside and PHA-L were purchased from Sigma. The lectin Griffithsin was obtained from the NIH AIDS Reagent and Reference Program. The lectins GNA and BanLec were isolated through previously described methods (van Damme et al., 1987). Isolation of recombinant BanLec from *E. coli* is described below.

Construction and mutation of BanLec expression vectors

A cDNA encoding a codon-optimized BanLec for expression in *E. coli* was generated from the protein sequence gi71042661 by Genscript. The cDNA was then cloned into the *E. coli* expression vector pET24b (Novagen) to be in frame with a 6x His-tag placed at the C-terminus . Site-directed mutations were introduced by the QuikChange Multi Site-Directed Mutagenesis Kit or by the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene). PCR primers for introduction of the desired mutations were designed using the QuikChange Primer Design Program; available at http://www.stratagene.com/sdmdesigner/default.aspx.

Purification of recombinant BanLec and mutants

A plasmid containing either the WT or a mutant form of BanLec was used to transform MDS 42 T7 or RosettaBlue pLysS *E. coli* cells. An overnight culture was used to inoculate 2 x YT medium. When the OD_{600} of the culture reached 0.7 -1.0, protein expression was induced with IPTG at a final concentration of 1 mM. Five hours post-induction, the bacteria were harvested, suspensions centrifuged, and cell pellets were frozen and stored at -20 °C until further processing. Recombinant protein was isolated by resuspending the pellet in 5 ml of 50 mM Tris, 0.5 M NaCl, and 0.02 % NaN_3 at pH 8.0 (the buffers will be referred to hereafter as IMAC-#, where # represents the amount of imidazole in mM) per 100 mL of culture grown. Lysozyme and DNAse I were added to reach concentrations of 1 mg/mL and 5 μg/mL,

respectively. The mixture was incubated at room temperature for 30 minutes with constant stirring. After the incubation, an equal volume of IMAC-50 buffer was added and the mixture was chilled on ice. Cells were further lysed with four rounds of 30 seconds of pulsed sonication at the 50% duty at power level 5 while on ice, followed by a one minute rest period between each 30 seconds of sonication. The insoluble material was pelleted by centrifugation at 10,000 x g for 20 minutes.

The resulting cleared lysate was added to Ni-NTA agarose (Qiagen) that had been equilibrated with IMAC-25 buffer. The suspension of lysate and resin was incubated for one hour at 4 °C with orbital rotation and then placed onto a column. The column was returned to room temperature, and the lysate was allowed to pass through the column via gravity. The column was then washed with IMAC-25 buffer until the flow-through had an absorbance value at 280_{nm} less than 0.05. Elution of the protein was then performed with IMAC-250 buffer. The protein-containing solution was then dialyzed against PBS using Slide-alyzer dialysis cassettes with filters of a 10 kDa molecular weight cut-off (Pierce). Two 2-hour dialysis procedures were performed at 4 °C against a volume greater than 200 times that of protein sample followed by overnight dialysis. The solution was then sterile-filtered through a 0.22 μm filter, aliquoted, and stored at -80 °C prior to use, where it could then be stored again at 4 °C for short-term use. Protein content of solutions was quantified by BCA (Pierce) using bovine serum albumin protein as a standard.

Production of pseudotyped HIV

Virus was produced using previously described methods (Yang et al., 1999). Briefly, production of pseudotyped virus was performed by co-transfecting 293FT cells with a plasmid containing a proviral genome shortened by a deletion in the envelope gene along with a plasmid that expresses an HIV-1 envelope gene. The following morning, the medium was changed. Forty-eight hours post-transfection, the supernatant was collected and centrifuged at approximately 300 x g for five minutes to remove any contaminating cells. For NL4-3 virus production, 293FT cells were transfected with the pNL4-3 plasmid.

Virus was harvested as described above. Virus was quantified by determining titers with TZM-bl cells or by measuring p24 antigen by ELISA.

Assessment of anti-HIV activity

Assays testing the anti-HIV activity of WT and H84T BanLec in PBMCs were performed as described previously (Ferir et al., 2011). For the TZM-bl cell assays, to each well of a white 96-well plate 100 µL of a solution containing cells, resuspended at 1 x 10^5 cells/mL in DMEM medium with 25 mM HEPES and 10% FBS, was added. The next day, the medium was removed by aspiration and fresh medium containing lectin or PBS as a control was added to the plate at a concentration 2-fold higher than the final concentration. After 30 minutes of incubation, virus diluted with medium was added, and the cells were incubated for 48 hours at 37 °C. After the incubation, 100 µL of medium were removed and replaced with 100 µL of ONE-Glo™ Luciferase reagent (Promega) for determination of luciferase expression.

HCV Experiments

Human subjects. All protocols involving human tissue were reviewed and exempted by the Rockefeller University Institutional Review Board.

Cell culture. Huh-7.5 (Blight et al., 2002) were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mM non-essential amino acids (NEAA). Huh-7.5 cells stably transduced with irrelevant shRNA or shCD81 (Huh-7.5 $CD81^{lo}$) were grown in complete DMEM media supplemented with 6 µg/mL blasticidin (Witteveldt et al., 2009). Primary cultures of human fetal liver cells (HFLCs) were isolated and propagated as previously described (Andrus et al., 2011). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

HCVcc inocula. The bicistronic *Gaussia* luciferase reporter genomes (BiGluc-) of different cell culture-adapted HCV (HCVcc) genotypic chimeras were used for infection of Huh-7.5 cells, specifically

Con1(1b)/Jc1 (G2833C, T2910C, A4274G, A6558G, A7136C), H77C(1a)/JFH (T2700C, A4080T), J4(1b)/JFH (T2996C, A4827T), J6(2a)/JFH, J8(2b)/JFH, S52(3a)/JFH (A4550C), ED43(4a)/JFH1 (A2819G, A3269T), SA13(5a)/JFH1 (C3405G, A3696G), HK6a(6a)/JFH (T1389C, A1590C), and QC69(7a)/JFH (T2985C, C8421T) (Gottwein et al., 2009). Jc1FLAG2(p7-nsGluc2A) virus (Marukian et al., 2008) was utilized for infection of HFLCs. Virus stocks were prepared by electroporation of *in vitro* transcribed RNA into Huh-7.5.1 cells and titered by limiting dilution assay, as previously described (Lindenbach et al., 2005). For Jc1FLAG2 (p7-nsGluc2A) viral stocks were further concentrated using Amicon Ultracel-100K filters (Millipore).

Cells were incubated with virus inoculum at a multiplicity of infection (MOI) of 0.1. Unless noted otherwise, the virus-lectin mixture was transferred to cells seeded in 96-well plates (Huh-7.5, 6.4×10^3 cells per well; HFLCs, $1x10^5$ cells per cm^2 in BD-Biocoat Collagen 1-coated plates), and incubated at 37 °C for 6 hours (h). Control antibodies against CD81 (2 µg/mL (JS81 clone); BD Pharmingen) and genotype 1a E2 (2 µg/mL (AR4A; (Giang et al., 2012)) were utilized. Cells were washed three times and further incubated at 37°C. HCVcc infections were terminated after 72 h. *Gaussia* luciferase secreted within the supernatants was quantified using the luciferase assay system (Promega, Madison, WI). All data was analyzed and plotted using GraphPad Prism 5.0 software. Effective concentration (EC) was determined by non-linear regression statistics.

Replication analysis. *In vitro* transcribed RNAs were electroporated into cells utilizing a 2 mm gap cuvette, as previously described (BTX ElectroSquare Porator ECM830; Harvard Apparatus, Holliston, MA (Lindenbach et al., 2005)). Virus-containing cells were mixed with mock electroporated cells at a 1:5 dilution and seeded in 96-well plates (12×10^3 cells per well). At 12 hours post electroporation (hpe), cells were treated with 265 nM H84T, D133G/38A, or 2′C-methyladenosine (2’ CMA; 10x EC50 previously reported (Lindenbach et al., 2005)). Replication kinetics were analyzed by harvesting supernatants at the indicated time points in 1X *Renilla* Luciferase Assay Lysis Buffer (Promega).

Quantification of *Gaussia* luciferase activity was performed on a Centro LB960 luminometer (Berthold Technologies, Oak Ridge, TN) using *Renilla* luciferase substrate (Promega) following the manufacturer's instructions.

Assessment of anti-influenza activity using pseudotyped lentivirus

A test system (Temperton et al., 2007) was used in which a retroviral core is pseudotyped with the hemagglutinin of the influenza virus in question. Pseudotyped lentiviral vectors that transduce a luciferase reporter gene were produced, as previously described, by calcium phosphate mediated transfection (Yang et al., 2007). Hemagglutinin and neuraminidase expression plasmids (Yang et al., 2007; Wei et al., 2010) were obtained from Gary Nabel (Vaccine Research Center, NIH). H1N1 pseudotypes were produced using pVRC-7730 (A/South Carolina/1/18(H1N1)HA-wt) and pVRC-9259 (A/Brevig Mission/1/18(H1N1) NA). H5N1 viruses were produced using pVRC 7705 (A/Thailand/1(KAN-1)/2004/ (H5N1) HA-wt) and pVRC -7708 (A/Thailand/1(KAN-1)/2004 NA/h). 293T cells in a 10 cm dish were co-transfected with 800 ng of HA plasmid, 100 ng of NA expression vector, 6.9 µg of pCMV Sport/h TMPRSS2 (a human type II transmembrane serine protease TMPRSS2 expression vector for the proteolytic activation of HA), 12 µg of pCMVΔR8.2, and 7.1 µg of pHR′CMV-Luc. Forty-eight hours post-transfection, supernatants were harvested, filtered (0.45 µm), and used to infect fresh 293T cells.

The day before infection, 1.25 x 10^4 293T cells in 100 µL DMEM medium containing 10% fetal bovine serum and 50 µg/mL geneticin were added to each well in 96-well tissue culture plates. Dilutions of BanLec solutions were mixed with pseudotyped HIV-1 immediately before adding to cells. After two days, the culture medium was removed and the cells lyzed using Steady-Glo® Luciferase reagent (Promega). Cell lysates were transferred to a white opaque 96-well plate and the extent of luminescence was measured using a luminometer (Tecan). The amount of pseudotyped virus used in this experiment was selected by titration on 293T cells to assure that the luciferase activity was in the linear response range of the assay system. The data are shown relative to the amount of luciferase

activity produced in cells infected with pseudotyped lentiviral vectors pre-treated with control buffer (PBS). Error bars represent standard error from duplicate determinations.

Influenza virus experiments

Animals. Female 18-20 g BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA) for this experiment. The mice were quarantined for 72 hour before use and maintained on Teklad Rodent Diet (Harlan Teklad) and tap water at the Laboratory Animal Research Center of Utah State University.

Virus. Influenza A/WSN/HA(NC/2099-N225G)/1933 virus was used in the mouse experiments (Smee et al., 2008). The hybrid (recombinant) influenza A/WSN/33 (H1N1) virus containing the HA gene of influenza A/New Caledonia/20/99 (H1N1) was generated by reverse genetics as described previously (Hoffmann et al., 2000; Mishin et al., 2005). The Asp225Gly mutation was introduced in the HA gene encoding the HA1 subunit by PCR-based site-directed mutagenesis by Dr. Larissa Gubareva (CDC, Atlanta, GA). The recombinant virus was designated influenza A/WSN/33 HAnc-Asp225Gly (H1N1). The virus became lethal after 7 serial passages in the lungs of the animals. Virus was plaque purified and a virus stock was prepared by growth in MDCK cells.

Experimental design. Groups of 10 mice were anesthetized by IP injection of ketamine/xylazine (50 mg/kg and 5 mg/kg, respectively) prior to challenge by the intranasal (IN) route with approximately 1 x 10^2 (2x LD50) cell culture infectious doses (CCID50) of virus per mouse in a 50 µL inoculum volume (Smee et al., 2008). H84T Banlec (dissolved in PBS pH=7.8 and sterile filtered using a 0.22 µm filter) was administered IN under anesthesia (as described above) once daily for 5 days with 0.03 or 0.1 mg/kg doses beginning 4 hours post-virus challenge. Control groups included mice treated orally with 10 mg/kg/day oseltamivir twice per day for five days at 12 hour intervals, or placebo (15 mice) treated with physiological sterile saline (PSS) by the IN route. All mice groups were monitored for morbidity and mortality through day 21 post-infection.

Hemagglutination assay

The haemagglutinating activity of the lectin was determined by a 2-fold serial dilution procedure using formaldehyde-treated rabbit erythrocytes. The haemagglutination titer was defined as the reciprocal of the highest dilution still exhibiting haemagglutination.

Isothermal titration calorimetry

Binding constants of the lectins for methyl-α-D-mannopyranoside were determined by isothermal titration calorimetry using a MicroCal VP-ITC calorimeter (Micro-Cal, Northampton, MA, USA) at 25 °C. Data were analyzed using Origins Ver. 7 software supplied with the instrument. The lectin in PBS, generally at a concentration of approx. 0.2 mM, was titrated with the ligand at 20 mM in the same buffer. The titration volumes were adjusted so that the titration proceeded to at least a 10-fold molar excess of ligand over lectin monomers. The relatively low binding constants (K_a < 1000 M^{-1}) precluded obtaining full saturation or a complete sigmoidal titration curve, from which a definitive stoichiometry can be calculated; thus, the stoichiometry was fixed at 1 for curve-fitting to calculate K_a; setting this parameter between about 0.5 and 2-3 had little effect on the K_a-value obtained.

Assessment of mitogenic activity by BrdU incorporation

PBLs were isolated as previously described, and resuspended in IMDM medium containing 10% FBS (IMDM-10) at a concentration of 2 x 10^6 cells/mL (Swanson et al., 2010). 50 µL of cell suspension were added per well of a white 96-well plate followed by 50 µL of IMDM-10 medium containing lectin at various concentrations or PBS. The cells were incubated at 37 °C for three days prior to an 18 hour addition of BrdU. Proliferation was measured by BrdU incorporation, which was detected via a chemiluminescence-based ELISA (Cell Proliferation ELISA (chemiluminescent); Roche) as per the manufacturer's instructions. Mitogenic activity was quantified as a stimulation index, which is the signal

of the stimulated cells divided by the signal of the untreated control cells (RLU of treated PBL / RLU of untreated PBL).

Flow cytometry to measure cellular activation

The expression of the cellular activation marker CD69 was measured after a 3-day incubation of PBMCs with varying concentrations of MVN or CV-N at 37 °C. Briefly, after washing with PBS containing 2% FBS, cells were incubated with solution containing FITC-labeled anti-CD4 mAb in combination with PE-labeled anti-CD69 mAb for 30 min at 4 °C. For assessing non-specific (antigen-independent) background staining, cells were stained in parallel with Simultest Control IgG γ1/γ2a (BD Biosciences). Finally, the cells were washed, fixed with 1% formaldehyde solution, and analyzed with a FACSCalibur, resulting data were processed with CellQuest software and analyzed with the FLOWJO software.

Bio-Plex cytokine assay

PBMCs were cultured in the presence of several concentrations of lectin and culture supernatant was collected after 72 h. The cytokine production profile was determined by the Bio-Plex 200 system (Bio-Rad, Hercules, CA) and Bio-Plex Human Cytokine 27-plex assay according to the manufacturer's instructions. The 27-plex assay kit contains beads conjugated with mAbs specific for interleukin-1α (IL-1α), IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), interferon-γ (IFN-γ), interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage-inflammatory protein-1α (MIP-α), MIP-1β, platelet-derived growth factor-BB (PDGF-BB), regulated on activation, normal T-cell expressed and secreted (RANTES), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF). For each cytokine, nine standards ranging from 0.5 to 32,000 pg/mL were run in parallel and the minimum detectable dose was

between 0.5 - 5 pg/mL. Standard curves and the concentrations of the cytokines within the samples were calculated with the Bio-Plex Manager 4.1 software.

Vaginal HIV-1 transmission

BLT mice (Wahl et al., 2012) were anesthetized and received 75 µg of H84T BanLec (resuspended in PBS) vaginally in a volume of 20 µL. Ten minutes after application of the lectin, the mice were challenged vaginally by 175,000 TCIU of HIV-1 JR-CSF. Mice were bled weekly and the plasma was analyzed for the presence of viral RNA for six weeks as described previously (Denton et al., 2012).

Glycocluster assays

Neoglycoprotein (bovine serum albumin as carrier presenting 24-28 derivatives of mannose after covalent conjugation by applying an activation of p-aminophenyl derivatives with thiophosgene (McBroom et al., 1972) and checked for lectin reactivity (Gabius et al., 1988)) was adsorbed to the plastic surface of microtiter plate wells (0.5 µg) establishing the matrix for binding the biotinylated lectins, which was quantitatively assessed spectrophotometrically by applying the streptavidin/β-galactosidase conjugate and the chromogen chlorophenolred-β-D-galactopyranoside as described (André et al., 1999). Titrations of the extent of binding with increasing amounts of inhibitor were performed to determine the concentration that reduces the signal intensity of positive controls to 50% (IC_{50}-value). The glycoclusters (bivalent phenylenediamine-based glycocylophane (**1**) and terephthalamide-based compounds **2**, **3**, mannose-presenting tetra- to dodecavalent glycoclusters (**4**-**8**), and maltose-containing bi- to tetravalent glycoclusters based on propargyl-derivatized alcohols **9**-**11**) (André et al., 2003, 2009, 2010; Papadopoulos et al., 2012) were individually tested (further details are described in the legend for Table S3). Cell assays were performed with the human SW480 colon adenocarcinoma line processed in FACScan runs using the fluorescent streptavidin/*R*-phycoerythrin complex (Sigma; 1:40) as indicator (Kopitz et al., 2013). A non-cognate sugar (galactose) was used in

parallel as an osmolarity control. Treatment of cells for 24 h with 150 μM 1-deoxymannojirimycin was performed to increase presentation of high-mannose-type *N*-glycans. Routinely, aliquots of a cell suspension were analyzed in parallel in triplicate with positive and negative controls and up to five independent series, with standard deviations not exceeding 13.9 % after normalization of the data.

Crystallization, data collection, and structure determination

A solution of C-terminally His_6-tagged BanLec (WT and H84T mutant) was concentrated to approximately 5 mg/mL in buffer (10 mM HEPES, pH 7.5) containing 150 mM NaCl. Crystals appeared overnight in drops containing equal volumes of protein and well solution (10-20 % PEG 8000 and 50-200 mM potassium phosphate). For the structures of BanLec in complex with dimannoside (Sigma), crystals were soaked in solution containing 50 mM glycan for 2.5 hours. All crystals were cryoprotected in well solution containing 20 % glycerol prior to flash freezing in liquid nitrogen.

All data were collected at LS-CAT at the Advanced Photon Source at Argonne National Lab. Data for WT, WT+dimannoside, and H84T+dimannoside were collected on line 21-ID-D, while data for H84T were collected on line 21-ID-G. Both lines were equipped with Mar300 detectors. Data were processed and scaled with HKL2000 (Otwinowski and Minor, 1997). The structures were solved by molecular replacement with Phaser (CCP4 suite) (Mccoy et al., 2007) using the previously solved structure of banana lectin (2BMY) as a starting model. The structures of WT, WT+dimannoside, and H84T+dimannoside BanLec were refined using Buster (Bricogne G., 2011) with iterative rounds of fitting in COOT (Emsley et al., 2010). The structure of H84T was refined using REFMAC (Winn et al., 2003). Structures were validated with Molprobity (Chen et al., 2010), Parvati (Zucker et al., 2010), and whatcheck (Hooft et al., 1996). Ligand statistics were obtained from the Uppsala Electron-Density Server (Kleywegt et al., 2004). Data refinement and statistics are given in Supplemental Tables 4 and 5.

NMR spectroscopy

NMR spectra used for the assignment of the His-tagged BanLec mutant Y46K/V66D in solubility buffer (25 mM acetate, 50 mM KCl, 50 mM arginine, 50 mM glutamic acid, 1 mM EDTA, 2.5 mM NaN_3, 1.5 % Triton X-100, 10% D_2O, pH 5.3) were acquired at 313 K on a 600 MHz Varian spectrometer equipped with a triple resonance cryo-probe with pulse field gradient. HN, N, CO, CA and CB assignments were obtained using a TROSY version of the following set of tridimensional experiments: HNCO, HNCA, HN(CA)CO, HN(CO)CA, CBCA(CO)NH, HNCACB (Pervushkin et al., 1999; Sattler et al., 1999). All spectra were processed using NMRPipe (Delaglio et al., 1995) and analyzed using Sparky (Goddard, 2008). The assignment of the spin system was realized using the program MARS (Jung and Zweckstetter, 2004). Assignments were transferred onto the ^{15}N-^{1}H-HSQC spectra of WT BanLec by overlay in Sparky and further verified by comparison of a panel of ^{15}N-^{1}H -HSQC spectra of BanLec mutants, in which chemical shifts occurred in correspondence with the assigned mutation. ^{15}N relaxation rates were measured using standard pulse sequences (Farrow et al., 1994).

Double mutant (Y46K/V66D) design and assignment

Based on the crystal structures, we designed a Y46K mutant that disrupts a hydrophobic patch at the tetramer interface by substituting one of its hydrophobic side chains with a charged residue. While the Y46K mutant formed the expected dimer as measured by ^{15}N NMR spin relaxation, aggregation was still observed over time. A second mutation, V66D, therefore needed to be introduced to increase the long-term solubility of the lectin. This double mutant (Y46K/V66D) yielded a dimeric protein with low propensity for aggregation, as judged by measurement of ^{15}N relaxation data R2/R1 and signal intensity in ^{15}N-^{1}H HSQC spectra. This mutant was thus used to carry out multidimensional NMR assignment experiments (Sattler et al., 1999), allowing for ~93% of the backbone resonances to be assigned (Figure S5). As most of the resonances in the spectra were virtually identical, the transfer to the new double mutants was straightforward except for signals from the immediate vicinity of the sites of the two mutations (see Figures S3A and B), leading to a final overall assignment percentage of 89%. Of

essential importance in the context of this work, full assignment of the third Greek Key regions was accomplished, because the chemical shift perturbations due to the double mutation were clearly localized on the other side of the protein (Figure S6). The validity of assignment transfer was further rigorously confirmed by running an extensive program with mutations of residues in and around the sugar binding sites (A9G, D35E, K130R, D35K, F131Y, K130Q, N82T, A86G, D38G, K130G, T107S, V36G, A81G, D133E) (data not shown).

MD Simulations

All MD simulations were conducted using the Amber 12 package (Case et al., 2005) with the ff99SB*-ILDN force field (Hornak et al., 2006; Lindorff-Larsen et al., 2012). The initial coordinates were obtained from the crystal structure of the apo WT and H84T BanLec. Glu and Asp residues were kept unprotonated at the pH of the experimental condition and the pK_a values predicted by the program PROPKA (Bas et al., 2008). The initial structure was solvated in a truncated octahedral water box, whose size was chosen such that the boundary of the box was at least 10 Å away from any of the protein atoms. The procedure was applied so that the water boxes were as similar as possible in the cases of both proteins. Three or two Cl^- ions were added into the water box of WT or H84T protein, respectively, to neutralize the system according to the Coulombic potential distribution. The resulting water box was then subjected to energy minimization for 1000 steps using harmonic restraints with force constant of 500 kcal mol^{-1} $Å^{-2}$, followed by 1000 steps unrestrained minimization. The temperature setting of the optimized system was increased from 0 to 313 K and then equilibrated at 313 K for 1 ns under NPT ensemble. Finally, a trajectory was recorded for 500 ns at 313 K under the NPT ensemble. During the simulation, all bonds involving hydrogen atoms were constrained using the SHAKE algorithm (Ryckaert et al., 1977). The integration time step was set to 2 fs, and the nonbonded cut-off was set to 8 Å. The snapshot was stored every 5 ps. Simulations were performed in two GPU workstations, each equipped with four NVIDIA GeForce GTX780 cards (assembled by Colfax International, Sunnyvale, CA). The accelerated MD simulations were set up following the published

protocol (Pierce et al., 2012). Both the torsional potential and the whole potential were boosted. To calculate N-H dipolar order parameters, each MD snapshot was first superimposed onto the first snapshot using all CA atoms in the secondary structure elements, and then N-H vectors were extracted and order parameters were computed according to a published procedure (Brueschweiler and Wright, 1994).

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